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Biallelic germline nonsense variant of *MLH3* underlies polyposis predisposition

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Purpose: Some 10% of familial adenomatous polyposis (FAP) and 80% of attenuated polyposis (AFAP) cases remain molecularly unexplained. We scrutinized such cases by exome-wide and targeted methods to search for novel susceptibility genes.

Methods: Exome sequencing was conducted on 40 unexplained (mainly sporadic) cases with FAP or AFAP from Finland. The DNA mismatch repair (MMR) gene *MLH3* (MutL Homolog 3) was pinpointed and prompted a subsequent screen of ~1000 Swedish patients referred to clinical panel sequencing for colon tumor susceptibility.

Results: Three homozygous carriers of a truncating variant in *MLH3*, c.3563C>G, p.Ser1188Ter, were identified among the index cases from the Finnish series. An additional biallelic carrier of the same variant was present in the Swedish series. All four patients shared a 0.8-Mb core haplotype around *MLH3*, suggesting a founder variant. Colorectal polyps from variant carriers showed no instability at mono-, di-, tri-, or tetranucleotide repeats, in agreement with previous findings of a minor role of *MLH3* in MMR. Multiple loci were affected by loss of heterozygosity, suggesting chromosomal instability.

Conclusion: Our results show that a biallelic nonsense variant of *MLH3* underlies a novel syndrome with susceptibility to classical or attenuated adenomatous polyposis and possibly extracolonic tumors, including breast cancer.

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INTRODUCTION

Familial adenomatous polyposis (FAP, MIM 175100) is characterized by multiple polyps in the colon and rectum and an increased risk of colorectal cancer. Most patients with classical FAP (polyp count at least 100) show heterozygous pathogenic germline variants in the *APC* gene.[1] Alterations in the extreme 5' or 3' ends or the alternatively spliced exon 9 of *APC* are associated with attenuated disease (AFAP, 10–100 polyps). (A)FAP-like polyposis may also result from pathogenic sequence changes in various other genes, including heterozygous variants in *POLE* and *POLD1* (polymerase proofreading-associated polyposis; MIM 615083 and 612591, respectively), or homozygous variants in *MUTYH* (MIM 608456), *NTHL1* (MIM 616415), and *MSH3* (MIM 617100) (ref. [1]). Significant proportions of polyposis cases (10–80% depending on polyp count) remain molecularly unexplained,[2] encouraging searches for novel susceptibility genes.

Accurate molecular and clinical classification of conditions with colorectal polyposis is a prerequisite for appropriate genetic counseling and testing to guide patient management and colorectal cancer prevention. To this end, we undertook an exome sequencing study of 40 index cases from a cohort with adenomatous polyposis from Finland in which pathogenic variants in known polyposis-associated genes had been

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excluded.[2] Results from this cohort, supplemented with panel sequencing data from some 1000 cases with various colorectal phenotypes from Sweden, unravel a novel polyposis syndrome associated with biallelic germline nonsense variant of *MLH3* (MutL Homolog 3).

MATERIALS AND METHODS

Patient cohorts and strategies to screen for pathogenic germline variants

The patient series investigated are described in Supplementary Materials and Methods and schematically depicted in Fig. **1a**. In the first phase, blood DNAs from 40 unrelated cases/families with adenomatous polyposis from Finland, without any detectable pathogenic sequence changes in known polyposis-associated genes[2] and in most cases sporadic at presentation, were submitted to exome sequencing (ES). Based on polyp count (with 100 polyps as a divider), 14 represented FAP and 26 AFAP. Guided by the findings from the Finnish polyposis cohort, 829 patients from Sweden, referred to Cancer Genetics Counseling clinics for a suspected hereditary colorectal cancer syndrome, were analyzed for *MLH3* variants from panel sequencing data. Variants with allele frequency below 0.003 (gnomAD), nonsynonymous (frameshift, stop gained/lost, missense, disrupting donor/acceptor site variants), and predicted pathogenic with at least five of six programs assessing protein function in silico were selected. The recurrent *MLH3* c.3563C>G, p.Ser1188Ter variant was further characterized by studying constitutional DNA (haplotype analyses for shared versus independent origin) and RNA (primer extension and cloning analyses for nonsense-mediated RNA decay). Additionally, tumor tissues from variant carriers were examined for microsatellite instability (MSI), loss of heterozygosity (LOH)/allelic imbalance (AI), CpG island methylator phenotype (CIMP), and MMR protein expression (see Supplementary Materials and Methods). Written informed consent preceded study participation and sample donation. This study was approved by the institutional review board of the Helsinki University Central Hospital (Helsinki, Finland) and by the local ethics committees of the Universities of Gothenburg and Lund, Sweden.

RESULTS

Identification of a recurrent germline variant in *MLH3*

Forty index cases with unexplained adenomatous polyposis from Finland were investigated by exome sequencing to dissect the underlying genes (Fig. **1a**). After filtering out variants common in the population and those unlikely to be pathogenic (see "Materials and methods"), a recurring nonsense variant affecting the DNA mismatch repair (MMR) gene *MLH3* (c.3563C>G, p.Ser1188Ter) (Table S1) was observed in four families (Fig. **1b**). The index individuals from families 158, 168, and 177 were homozygous for the variant (Figure S1). Polyposis was attenuated in the first one and profuse in the latter two. The index person from family 1007 was heterozygous for the variant and showed mild polyposis. The variant is absent in disease- or locus-specific databases and rare in the population (allele frequency 0.0002603 in gnomAD and 0.00238 in Finns based on SISu; no homozygotes reported).

Our subsequent literature search identified a recent study on 91 Swedish cases referred to clinical panel sequencing for FAP or Lynch syndrome (LS, MIM 120435 and related).[3] A sporadic case with apparently classical FAP (case IV:69 [ref. [3]], who is the index individual of SWE family in Fig. **1b**) was diagnosed with the homozygous *MLH3* p.Ser1188Ter variant. Prompted by this finding, the entire *MLH3* gene was investigated in an additional cohort of 829 Swedish cases referred to clinical laboratory screening for a suspected hereditary colorectal cancer syndrome (Fig. **1a**). No additional cases with homozygous pathogenic or likely pathogenic *MLH3* variants were found, and no concomitant nonsynonymous variants in *MLH3* were detected in any of the patients (Table S2). Despite looking like unrelated families, the four Finnish cases and the single Swedish case with the *MLH3* p.Ser1188Ter variant all shared a 0.8-Mb haplotype around *MLH3* (Fig. **2a**), suggesting origin from a common ancestor. The variant was absent in cohorts of nonpolypotic colon cancer (Fig. **1a**).

Molecular characteristics of the *MLH3* founder variant

The *MLH3* p.Ser1188Ter variant causes an immediate stop of translation, leading to the loss of the MLH1 binding domain of MLH3 (Fig. **2b**). The variant was associated with nonsense-mediated RNA decay by primer extension and cloning experiments (Figure S2). While none of the commercially available MLH3 antibodies tested for immunohistochemistry produced staining patterns of sufficient specificity, MSI results were unequivocal: no single tumor (adenoma or carcinoma, among a total of eight tumors investigated) from the *MLH3* p.Ser1188Ter variant carriers revealed any MSI at mono-, di-, tri-, or tetranucleotide repeats, either by conventional polymerase chain reaction (PCR) (Fig. **2c**) or small pool PCR. Instead, 8 of 15 di-, tri-, and tetranucleotide markers tested showed loss of heterozygosity or allelic imbalance in colorectal and other tumors (including breast carcinoma), suggesting chromosomal instability (Fig. **2c**). Immunohistochemical analysis showed normal expression of MLH1 protein, the binding partner of MLH3 (ref. [4]). One adenoma of three tested revealed a *BRAF*-V600E variant in association with CpG island methylator phenotype (see Supplementary Materials and Methods).

Concurrent sequence changes in other genes from *MLH3* variant carriers

Apart from the biallelic p.Ser1188Ter variant in *MLH3*, the index patient from family 158 showed two *MSH3* variants, a truncating (c.1308_1309delAG, p.Glu437fs) and a missense change (c.2692A>C, p.Asn898His) (Table S1). However, a complementary DNA (cDNA) cloning experiment showed that both variants affected the same allele; moreover, MSH3 protein was present in tumor tissue by immunohistochemical analysis (Figure S3). High-penetrance heterozygous

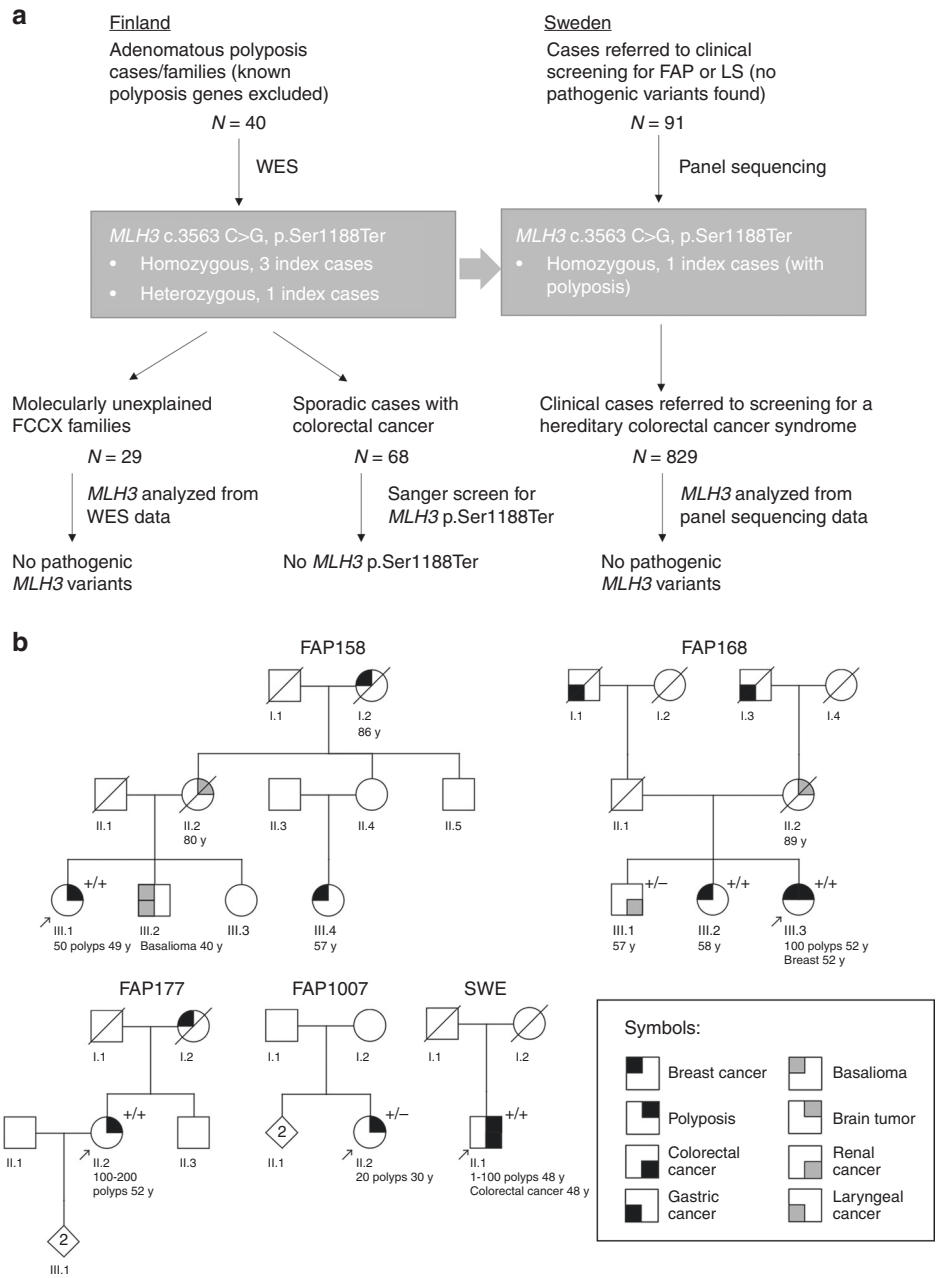

Fig. 1 Discovery of the pathogenic MLH3 variant in five polyposis families (**a**) Flow-chart of this investigation including the Finnish and Swedish arms. *MLH3* findings and methods used for their identification are shown. (**b**) Pedigrees of polyposis families with the *MLH3* p.Ser1188Ter variant. Families 158, 168, 177, and 1007 are from Finland and family SWE is from Sweden. The pedigrees were generated with Pedigree Chart Designer. Numbers below the symbols are patient identifiers. Arrow denotes the index person. Carrier status for the *MLH3* c.3563C>G, p.Ser1188Ter variant is shown (+/+, homozygous carrier, +/− heterozygous carrier). Tumor manifestations and age at diagnosis (years) are given below the patient symbol. *AFAP* attenuated familial adenomatous polyposis, *ES* exome sequencing, *FAP* familial adenomatous polyposis, *LS* Lynch syndrome.

pathogenic germline variants of *MSH3* are very rare in individuals with a suspected cancer predisposition.[5] Furthermore, family 158 with microsatellite-stable tumors showed no evidence of a possible synergistic effect with other MMR gene variants, as proposed to occur in some Lynch families.[6] Therefore, heterozygosity for the *MSH3* variants in family 158 is likely to represent a secondary finding. The index case from family 168 carried a potentially pathogenic[7] splice variant in *CHEK2* (c.319+2T>A), but it was absent in her affected siblings. The lack of additional convincingly or likely pathogenic germline variants by ES suggested that the biallelic *MLH3* p.Ser1188Ter variant was the primary alteration behind polyposis in families 158, 168, and 177.

MMR gene variants found in index cases without the *MLH3* founder variant

Based on clinical presentation as polyposis patients, previous diagnostic tests conducted on the 40 polyposis cases from

a

Marker	Location	FAP158		FAP168		FAP177		FAP1007		SWE		
cen	(GRCh37)											
D14S268	14:73271727-73271847	139	139	142	150	137	141	135	139	139	139	
D14S1025	14:74239463-74239609	143	143	143	143	143	143	143	143	143	143	
D14S1047	14:74898795-74898933	145	145	145	145	145	145	139	145	137	145	
MLH3 rs193219754 (exon4)	14:75506621	S1188*	S1188*	S1188*	S1188*	S1188*	S1188*	wt	S1188*	S1188*	S1188*	0.8 Mb
MLH3 rs28756981 (exon1)	14:75515668	C	C	C	C	C	C	A	C	C	C	
D14S284	14:75708386-75708541	154	154	154	154	158	154	168	154	154	154	
D14S273	14:75777880-75778015	130	130	132	132	130	132	130	138	134	134	
D14S61	14:76335308-76335522	158	166	158	162	150	158	140	164	158	158	
D14S263	14:77329474-77329617	146	146	146	146	146	148	146	146	N/A	N/A	
D14S1008	14:79901912-79902029	120	120	118	120	120	120	116	120	120	120	
D14S1000	14:82105927-82106058	122	122	122	126	122	122	124	126	128	128	
tel												

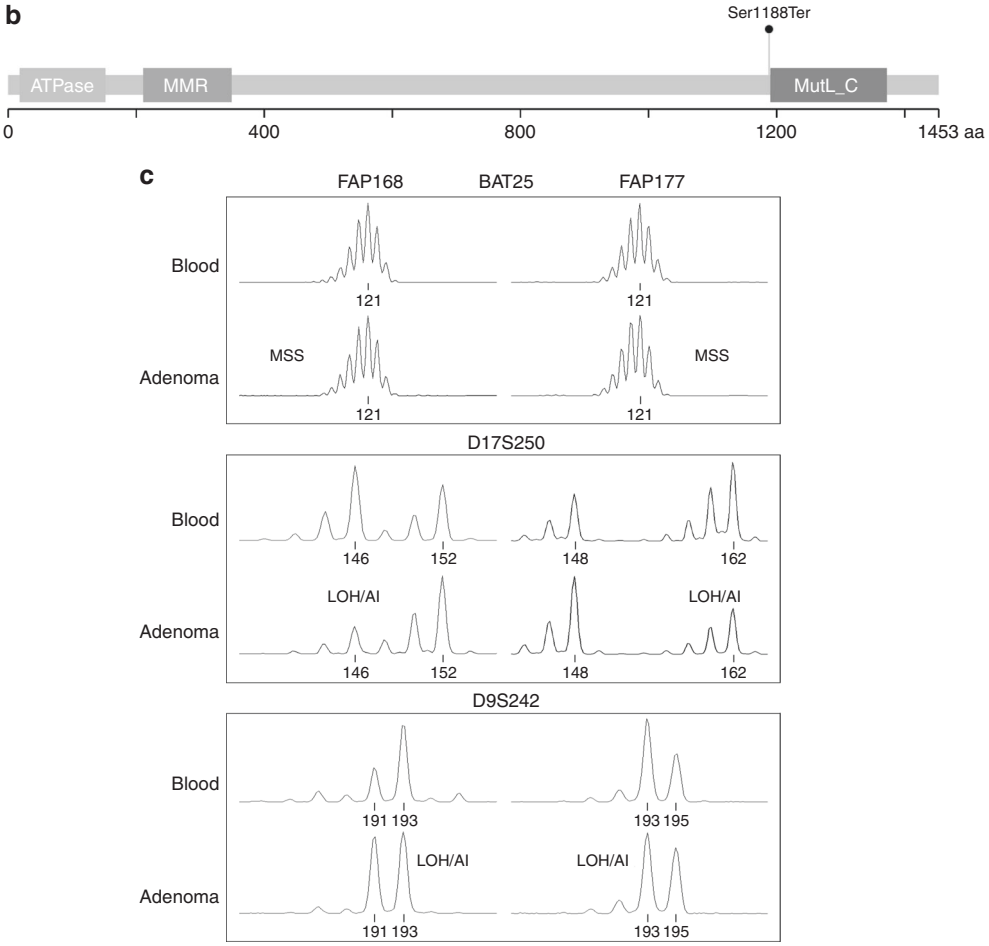

Fig. 2 Origin and molecular characteristics of the MLH3 p.Ser1188Ter variant (**a**) Shared founder haplotype in carriers of the *MLH3* p.Ser1188Ter variant. The haplotypes for an 8.8-Mb region between microsatellite markers D14S268 and D14S1000 are shown for the index patients from the indicated families (158, 168, 177, and SWE, homozygous carriers; 1007, heterozygous carrier). The conserved disease haplotype associated with the p.Ser1188Ter variant (between positions 73,271,847 and 75,777,880) is shaded. Deviation from the conserved haplotype at D14S1047 in SWE could result from either mutation or recombination. A core haplotype of 0.8 Mb (between D14S1047 and D14S284) that includes the *MLH3* gene and is shared by all p.Ser1188Ter variant carriers is indicated by brackets. (**b**) Location of the p.Ser1188Ter variant relative to the main functional domains of MLH3. A lollipop diagram of the MLH3 protein was created by MutationMapper. The functional domains are as follows: ATPase domain, MMR (DNA mismatch repair domain), and MutL_C (MutL C terminal dimerization domain). (**c**) Results from microsatellite instability (MSI) and allelic imbalance (AI)/loss of heterozygosity (LOH) analyses of colorectal adenomas from *MLH3* p.Ser1188Ter variant carriers. The markers used were BAT25 (mononucleotide repeat from chromosome 4), D17S250 (dinucleotide repeat from chromosome 17), and D9S242 (tetranucleotide repeat from chromosome 9). All tumors were microsatellite-stable (MSS, marker BAT25). Allelic imbalance was evident with D17S250 and D9S242.

Finland focused on established polyposis-associated genes. Our exome sequencing experiments identified rare heterozygous variants in the LS predisposition gene *MSH2* in two cases. Both variants involved the evolutionarily conserved MSH3/MSH6 interaction domain of the *MSH2* gene product and were absent in disease- or locus-specific databases. The index individual from family 1001 (with polyp count 10 and a personal and family history of colorectal cancer) had a 27-bp in-frame deletion (c.1140_1166del, p.Leu381_Arg389del) likely to have pathogenic significance because it was accompanied by MSI-high and absent MSH2 and MSH6 proteins in tumor tissue. The index individual from family

1025 (a sporadic case with polyp count 20–30) revealed a missense change (c.1337A>G, p.Asp446Gly) predicted deleterious by all six in silico programs tested. However, as the variant was associated with stable microsatellites and normal MMR protein expression in tumor tissue, its significance remains unknown.

DISCUSSION

We describe a novel polyposis and cancer syndrome associated with biallelic (homozygous) pathogenic germline variant of the "minor" MMR gene *MLH3*. *MLH3* was originally identified as a colon tumor susceptibility gene in mice some 20 years ago.[8] A few dozen heterozygous germline variants in *MLH3* with possible pathogenicity, mostly missense but also truncating, are listed in the InSiGHT database (www.insight-group.org). The clinical significance of such variants remains unsettled; they may play a role in familial nonpolypotic colon cancer or LS predisposition (MIM 614385) (refs. [9,10]). MSI findings from tumor tissues vary from instability at primarily di- and tetranucleotide repeats[9] to no MSI.[10] Cosegregation results are variable and a role as a low-penetrance colon cancer susceptibility gene has been suggested.[10,11] Due to the lack of genome-wide sequencing data, concurrent pathogenic variants in other cancer-relevant genes cannot often be excluded. No increased risk of colorectal adenomas in putative LS families with *MLH3* variants has been reported.[9,10]

Our results link a biallelic germline nonsense variant of *MLH3* to a clinical and molecular phenotype different from LS: microsatellite-stable adenomatous polyposis exhibiting chromosomal instability. Whether or not the rate of LOH/AI in *MLH3* variant carriers exceeds that observed in the corresponding sporadic tumors remains to be addressed by future studies. Moreover, the somatic *BRAF*-V600E variant was detected in one of three adenomas from *MLH3* p.Ser1188Ter variant carriers, which is another discriminating feature since *BRAF*-V600E generally predicts the absence of LS.[12] The lack of MSI in the *MLH3* variant carriers is compatible with the reported minor role for the MLH1-MLH3 complex (hMutLγ) in MMR, reflecting redundancy with the main MMR complex MLH1-PMS2 (hMutLα).[4] The accumulated data from yeast, mouse, and human cells suggest a primary function for MLH3 in meiotic recombination instead of MMR.[13–15] Extrapolation from literature data allows us to hypothesize that relevant functions that may fail in the *MLH3* p.Ser1188Ter-associated tumorigenesis might involve, for example, DNA damage response (defective in *Mlh3*−/− mice)[14] or recombination-related processes.[16]

LS associated with "major" MMR genes *MLH1*, *MSH2*, *MSH6*, and *PMS2* can occasionally present with polyposis. Two index cases from the Finnish polyposis series (2/40, 5%), both with attenuated polyposis, revealed novel MMR gene variants in *MSH2* by exome sequencing. The findings comply with Kalady et al.[17] and suggest that although ten or more adenomas prompt testing for polyposis, the possibility of LS should not be overlooked.

While heterozygous sequence changes of *MLH1*, *MSH2*, *MSH6*, and *PMS2* can cause predisposition to LS, biallelic inactivation of the same genes underlies constitutional MMR deficiency syndrome (CMMRD; MIM 276300). CMMRD is characterized by variable penetrance and diverse clinical manifestations, including colonic adenomatous polyposis of variable degree.[18] Clinically, the *MLH3*-associated syndrome we describe shares certain CMMRD features in analogy to the *MSH3*-associated polyposis syndrome identified recently.[5] The age at onset of polyposis (classical or attenuated) was relatively late (48–52 years in the biallelic index cases), which might suggest reduced penetrance. The cases were considered sporadic relative to polyposis, but the family histories did include various noncolonic cancers, especially breast cancer (Fig. **1b**). Breast cancer co-occurred with polyposis in the index patient (III.3) from family 168 and was the only tumor manifestation in her sister (III.2), likewise homozygous for the *MLH3* founder variant. While the possibility of the (post-menopausal) breast carcinomas being phenocopies cannot be excluded, heterozygous (truncating) germline variants in *MLH3* identified by recent massive parallel sequencing studies of breast cancer patients support an association between *MLH3* and breast cancer.[19] The observation of brain tumors in the mothers of the index cases of families 158 and 168 is also of potential interest given that multiple *MLH3* variants have been reported to coexist in colon cancer patients from families with LS-associated brain tumors.[20] The fact that none of the parents of our homozygous polyposis patients were affected with polyposis is consistent with recessive inheritance; however, two proven heterozygous individuals had a disease phenotype (attenuated polyposis in II.2 from family 1007 and renal urothelial cancer in III.1 from family 168). Because our data rely on a founder variant enriched in the Finnish population, investigations on additional cohorts and populations are warranted to establish the significance of *MLH3* as a polyposis-predisposing factor and to define the tumor spectrum of the syndrome.

SUPPLEMENTARY INFORMATION

The online version of this article (https://doi.org/10.1038/s41436-018-0405-x) contains supplementary material, which is available to authorized users.

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DISCLOSURE

The authors declare no conflicts of interest.

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